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The International Arabic Journal of Antimicrobial Agents
ISSN: 2174-9094

2019
Vol. 9 No. 2:4
doi: 10.3823/834

Intestinal colonization of infants with multidrug resistant *Pseudomonas aeruginos* in tertiary care center in Jordan

Noor Issam Shishtawi[1], Manar Al-lawama[2], Asem A. Shehabi[1]

1 Department of Pathology-Microbiology and Forensic Medicine, The University of Jordan, Amman, Jordan.
2 Department of pediatrics, Jordan University Hospital, Amman, Jordan.

Contact information:

Professor Asem A. Shehabi.

asashehabi2@gmail.com

Abstract

Background: *Pseudomonas aeruginosa* is among the most common opportunistic hospital pathogens, which exhibit an innate resistance and has developed increasing resistance to many useful antimicrobial agents over the last decades. This study investigated the occurrence of important types of ESBLs and MBLs in association with potential important virulence factors among *P. aeruginosa* isolates from feces of Jordanian infants.

Methods: A total of 302 feces samples were obtained randamely from neonates and infants admitted to Pediatric Clinic and the Neonate Intensive Care Unit (NICU)/Jordan University Hospital (JUH), over a 9-month period of 2016-2017. Fecal samples were cultured for *P. aeruginosa* and their growth was identified and tested using microbiological and antibiotic susceptibility methods. Additionly, virulence factors, antimicrobial resistance genes and genotypes were detected using Polymerase Chain Reaction (PCR).

Results: A total of 16/302 (5.3%) of *P. aeruginosa* isolates were recovered from feces samples of only hospitalized infants. Antimicrobial susceptibility of the isolates ranged between the lowest 18.7% to meropenem and highest of 87.5% to azetreonam among 9 tested drugs. The percentage of specific genes of *ESBLs* and *MBLs* in 16 *P. aeruginosa* isolates were the following: *blaOXA-50, blaTEM, blaCTX-M, blaVIM, blaKPC, blaSHV, blaGES,* and *blaVEB* were detected at the rate of 13 (81.2%), 13 (81.2%), 12 (75%), 12 (75%), 11 (68.7%), 10 (62.5%), 2 (12.5), 1 (6.2%), respectively. The percentage of the potential virulence genes in the same isolates were detected as follow: *las*B, *alg*D, *tox*A, *exo*S and *exo*U at the rate of 100%, 87.5%, 81.2%,

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81.2%, 31.2%, respectively. All *P. aeruginosa* isolates observed to develop beta-hemolysis on both human and sheep blood agar, and to produce either pyoverdin (56.3%) or pyocyanin (43.7%).

Conclusions: The present study demonstrates high occurrence of multidrug resistant *P. aeruginosa* isolates from only hospitalized infant feces which also carried high rates of important genes of *ESBLs* and *MBLs* and potential virulence factors.

Keywords
Pseudomonas aeruginos; Multidrug resistance; Intestinal tract; Infant; Virulence factors.

Received 15-06-2019; accepted 17-07-2019

Introduction

Pseudomonas aeruginosa is a major cause of nosocomial infections in children and adults, and its responsible for about 10% of all hospital-acquired infections worldwide [1, 2].

P. aeruginosa causes severe infections especially in immune-compromised patients, and it continues to pose a therapeutic challenge resulting in high rate of morbidity and mortality due to development of drug resistance during antibiotic treatment of patients [3-4].

P. aeruginosa infections are becoming more difficult to treat, and the number of multidrug drug resistant isolates from clinical and hospital environment sources is increasing worldwide including Arab Middle East countries [1, 5-7]. Recently, the most important issue is the emergence of carbapnemases in *P. aeruginosa* which considered as the last-line agents against Gram-negative bacteria infections [8].

P. aeruginosa infections are especially difficult to prevent and to treat because of its common occurrence in hospital environment and water sources, and for both intrinsic resistance to many antibiotics as well as rapid development of antibiotic resistance [9-11]. These features facilitate *P. aeruginosa* infections to become a serious health care issue in hospitals worldwide [12].

P. aeruginosa is associated with presence and execration of large virulence factors that are involved in various stages of the infection process allowing the organism to colonize any part of the infected host. These virulence factors include flagellum, type IV pili, lipopolysaccharide, a type III secretion system, and alginate which are involved in the adhesion, motility and colonization [13-14]. In particular, *P. aeruginosa* is possessing cell-associated and extracellular virulence factors controlled by a complex regulatory circuit involving cell-to-cell signaling (quorum sensing) system, allowing host-pathogen interactions during infections [15]. The organism also releases several metabolites including mainly, exotoxins, exoproteases, heamolysins and pyocyanin or pyoverdin-fluorescein pigments. All these virulence factors are causing extensive tissue damage as well as facilitate bacterial multiplication and the spread in host tissues [5, 16].

The emergence of extended-spectrum-β-lactamases (ESBLs) and metallo-β-lactamases (MBLs) compromised the effect of most β-lactam antibio-

tics including carbapenems which are the drug of choice for treatment of infections of Gram-negative ESBL-producers [17-18]. Reports of carbapenem resistant *P. aeruginosa* recovered from patients have been documented from different Arab regions, including Jordan, Syria, Egypt, Saudi Arabia, Kuwait and Lebanon [5, 19-23].

This study aims to investigate the distribution of most important types of ESBLs, MBLs, and potential virulence genes in *P. aeruginosa* isolates from feces of examined infants in a tertiary care center in Amman, Jordan by using culture and molecular PCR methods.

Materials and Methods

This prospective study was randamly conducted at Jordan University Hospital (JUH) neonatal unit and at outpatient pediatric clinic over the period from May 2016 to February 2017, only one sample was collected from each patient.

The study was approved by the IRB and ethical committee at JUH (2016/118). Informed consent was taken from parents before obtaining the fecal samples.

Neonates and infants up to one year old were included in the study. Neonates and infants with gastrointestinal anomalies and those whoes parents declined consent were excluded. Anal swabs were colleted by neonatal nurse or physician.

Medical charts of included infants were reviewed and their parents were interviewd using a structured data sheet. Demographic and clinical data in addition to current or recent antibiotics therapy within two weeks of the encounter were also documente.

Bacterial standard strains

The following bacterial standard strains were included as control: *P. aeruginosa* controls: ATCC 27853, ATCC 9027, and *P. aeruginosa* PAO1 strain had been used for quality control of antimicrobial susceptibility test and PCR detection of *P. aeruginosa* strains, and controls (CL 120 & CL 162, Universite´ Libanaise, Tripoli, Libanon, Prof. Monzer Hamze) that were positive for IMP-1, VIM-2, *K. pneumonia (*ATCC BAA-1705) positive for blaKPC and *E. coli (*ATCC 51446) positive for blaCTX-M, and blaNDM-1).

Culture, isolation, identification

All fresh collected fecal samples had been diluted in 2 ml NaCl-saline (0.9%) and then inoculated onto Cetrimide *Pseudomonas* Selective Agar (Merck, Germany).

All suspected growth of *P. aeruginosa* was identified as *P. aeruginosa* according to the following characteristics: positive oxidase test, growth at 42 °C, negative lactose and glucose fermentation in tube of Kligler iron agar. Five colonies of *P. aeruginosa* growth were subcultured into cetrimide agar to get pure culture of the organism and then a few colonies were inoculated and stored in cryotubes containing brain-heart infusion broth with 20% glycerol at -70 °C for further investigation and confirmation by PCR.

Antimicrobial susceptibility test using disc diffusion method

Antimicrobial susceptibility test using disc diffusion method was performed according to the recommendation of the Clinical Laboratory and Standards Institute (CLSI, 2015) [24].

Minimum inhibitory concentration (MIC) using E-test

All isolates of *P. aeruginosa* which were MDR to three or more antibiotic classes have their MICs measured by E-test for ceftazidime, amikacin, colistin, and imipenem, and their results were interpreted according to CLSI, 2015 [24].

DNA extraction and PCR procedure

The bacterial DNA was extracted using the Wizard genomic DNA Purification Kit

(Promega, USA) according to manufactures instructions. Two PCR assays were performed; one is specific for the genus *Pseudomonas*, while the other is specific for *P. aeruginosa*. Two pairs of primers were used for each assay based on 16 ribosomal DNA (rDNA) sequence. A positive control of *P. aeruginosa* ATCC 27853 and ATCC 9027 were used for the identification of the specific sequences.

Plasmid extraction

The bacterial plasmid was extracted using the EZ-10 Spin Column Plasmid DNA- Minipreps Bio Basic kit (Canada) according to manufactures protocol.

Confirmation of P. aeruginosa

All isolates were confirmed as *P. aeruginosa* using specific primers and PCR as shown in **Table 1**.

Table 1. Primer targets, sequences and their product size for five ESBLs and virulenc genes among *P. aeruginosa* isolate.

Target	Primer	Primer sequence (5' to 3')	Product size (bp)	Ref.
Pseudomonas species	PA-GS-F PA-GS-R	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	25
P. aeruginosa	PA-SS-F PA-SS-R	GGGGGATCTTCGGACCTCA TCCTTAGAGTGCCCACCCG	956	25
blaCTX-M	CTX-M (F) CTX-M (R)	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550	26
blaVEB-1	VEB-1 (F) VEB-1 (R)	CGACTTCCATTTCCCGATGC GGACTCTGCAACAAATACGC	642	27
blaIMP	IMP-A IMB-B	GAAGGCGTTTATGTTCATAC GTACGTTTCAAGAGTGATGC	587	28
blaVIM	VIM2004A VIM2004B	GTTTGGTCGCATATCGCAAC AATGCGCAGCACCAGGATAG	382	28
blaSHV-1	SHV-1 (F) SHV-1 (R)	TGGTTATGCGTTATATTCGCC GCTTAGCGTTGCCAGTGCT	867	29
blaGES-1	GES-1 (F) GES-1 (R)	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGG	864	30
blaKPC	KPC (F) KPC (R)	ATGTCACTGTATCGCCGTCT TTACTGCCCGTTGACGCCC	880	31
blaOXA-50	OXA- (F) OXA- (R)	GAAAGGCACCTTCGTCCTCTAC CAGAAAGTGGGTCTGTTCCATC	400	32
blaNDM-1	NDM-1 (F) NDM-1 (R)	GGTGCATGCCCGGTGAAATC GAGCACTTCTTTTGTGATGGC	660	33
Tox A	Tox A-F Tox A-R	CTGCGCGGGTCTATGTGCC GATGCTGGACGGGTCGAG	270	34
Las B	Las B-F Las B-R	GGAATGAACGAAGCGTTCTCCGAC TTGGCGTCGACGAACACCTCG	284	34
Alg D	Alg D-F Alg D-R	CGTCTGCCGCGAGATCGGCT GACCTCGACGGTCTTGCGGA	313	34
Exo S	ExoS-F ExoS-R	CTTGAAGGGACTCGACAAGG TTCAGGTCCGCG TAGTGAAT	504	15
ExoU	ExoU-F ExoU-R	GGGAATACTTTCCGGGAAGTT CGATCTCGCTGCTAATGTGTT	428	15
PilB	PilB-F PilB-R	ATGAACGACAGCATCCAACT GGGTGTT GACGCGAAAGTCGAT	826	15

Detection of ESBLs, MBLs and virulenc genes

All primers, sequences and their product size for detection of extended-spectrum-β-lactamases (ESBLs), metallo-β-lactamases (MBLs) and virulenc genes were used according to references (25-34) as shown in **Table 1**.

Statistical analysis

Data generated from the study were tabulated as Microsoft Excel sheet and Uploaded to Statistical Package for Social Sciences (SPPSS version 20), frequency and percentage were calculated for the categorical data and Pearson's chi-squared test or Fisher's exact test were applied to determine potential factors associated with *P. aeruginosa* and to determine whether there are any statistical differences between groups, the level of significance was set at a P value of 0.05 to test the hypothesis of no association. Fisher's exact test replaces chi-squared test when the minimum expected count is less than five.

Results

General demographic characteristics of all 302 examined infants which were divided in hospitalized and non-hospitalized infants are shown in **Table 2**. A total of 16/302 (5.3%) isolates were found to be positive for *P. aeruginosa* **(Table 2)**. All positive *P. aeruginosa* isolates were found in hospitalized infants. The age range of the enrolled hospitalized infants being colonized with *P. aeruginosa* was (1-27) days, about 10 (62.5%) positive colonization occurred in females ($P = 0.026$) which is significant, positive colonization with

P. aeruginosa during the length of hospital stay had been taken into account, the mean length of hospital stay was 8.1 days **(Table 2)**. All *P. aeruginosa* isolates were resistant to ciprofloxacin, norfloxacin and imipenem (25%), followed by azetreonam (31%), meropenem (38%) piperacillin-tazobactam (44%9, ceftazidime and amikacin (56%) and gentamicin (75%) **(Table 3)**. The Minimum Inhibitory Concentration (MIC) range for four antibiotics are shown in **Table 3**, and none of the isolate was resistant to colistin.

The majority of fecal *P. aeruginosa* isolates were positive for the following virulence factors: ElastaseB (100%) and AlgD (87.5%), and slightly in less percentage (81.3%) for both extracellular protein toxins (exoenzymeS and exotoxinA) **(Table 4)**. Both ExoenymeU and PilB protein were only positive in 61.3% and 6.3%, respectiviely. All 16 isolates showed pigmentation of pyoverdin (57.3%) or pyocyanin (42.7%) and all produced beta-hemolysis on human and sheep blood in vitro **(Table 5)**. Among the ESBLs, TEM was the most frequent enzyme found in (81%) in *P. aeruginosa* isolates, followed by CTX-M (75%), whereas the other types of VEB and GES were detected in the range of 6 % and 13%, respectively. Both blaOXA-50 and KPC were found at 81% and 69%, respectively **(Table 6)**.

Table 2. Major demographic characteristics of 302 investigated infants.

Patients characteristics	hospitalized infants		Non -hospitalized infants		P-Value
	n	%	n	%	
Gender					
Males	104	60	66	51.1	0.006
Females	69	40	63	48.9	
Total	173	57.3	129	42.7	
Age					
1-29 days	170	98.3	43	33.3	< 0.001
1-12 months	3	1.7	86	66.7	
Clinical situation					
Presence of diarrhea	0		37	28.7	< 0.001
Prior use of Antibiotics					
Treatment with Antibiotics	61	35.3	34	26.4	0.049
No antibiotics treatment	112	64.7	95	73.6	

Table 3. Antimicrobial susceptibility pattern of 16 *P. aeruginosa* isolates using diffusion disc amd MIC-Etest mehods.

Domains/Facets	Resistant		MIC50	MIC90	MIC Range
	No.	%	µg/ml	µg/ml	µg/ml*
Ciprofloxacin	4	25	-	-	-
Imipenem	4	25	3.6	6.5	0.75-32
Aztreonam	5	31	-	-	-
Meropenem	6	38	-	-	-
Piperacillin-tazobactam	7	44	-	-	-
Ceftazidime	9	56	1.92	3.45	0.50-12**
Amikacin	9	56	7.62	13.72	3-48
Gentamicin	12	75	-	-	-
Colistin	-		0.18	0.32	0.50-1.2

*: Breakpoints for susceptible P. aeruginosa isolates (µg/ml) were the following: Amikacin; ≤ 8, Ceftazidime; ≤ 8, Imipenem ≤ 4, Colistin; ≤ 2.
**: Only 25% of isolates were resistant using Etest.

Table 4. Distribution of virulence genes among 16 *P. aeruginosa* isolates.

Virulence factor	Gene	No. (%) Positive virulence genes	
Elastase B	las B	16	(100)
Alginate	alg D	14	(87.5)
Exotoxin A	tox A	13	(81.3)
Exoenyme S	exo s	13	(81.3)
Exoenyme U	exo u	5	(31.3)
PilBprotein	pil B	1	(6.3)
Pyoverdin*	-	9	(57.3)
Pyocyanin*	-	7	(42.7)
Beta-hemolysis**	-	16	(100)

*: Recorded by presence the yellow-green color (pyoverdin) or blue-green color (pyocyanin) of isolates on Pseudomonas culture agar after 24-48 hr incubation
**: Detected by presence of complete hemolytic activity of isolat es on human and sheep blood agar plates after 48 hr incubation

Table 5. Distribution of ESBLs genes among 16 *P. aeruginosa* isolates.

Virulence factor	*P. aeruginosa* isolates	
	No.	%
blaTEM	13	81
blaCTX-M	12	75
blaSHV-1	10	63
blaGES-1	2	13
blaVEB	1	6
blaKPC	11	69
blaOXA-50	13	81
blaVIM	12	75
blaIMP	Nil	
blaNDM-1	Nil	

Discussion

Pseudomonas aeruginosa is accounting for 7.1% of all hospital infections in the United States according to a recent study [35]. The organism is a frequent colonizer of intestinal tract of human, especially after prolonged antibiotic treatment or ICUs admission. Since *P. aeruginosa* is widely distributed in most hospital settings, the organism is often colonizing and infect hospitalized patients, particularly immune-compromised patients, patients with compromised lungs and burns as well as infants in neonatal intensive care units (NICU) [36, 39-40]. Previous studies have demonstrated that colonization of adults and children patients with *P. aeruginosa* can be associated with diarrhea or subsequent infection with the same strain of *P. aeruginosa* [36, 39-40]. Therefore, detection of patients in the ICUs colonized with *P. aeruginosa* can be helpful to control hospital acquired infection and in selection of empiric antibiotics in suspected cases of sepsis [36].

The present study shows that 5.3% of *P. aeruginosa* isolates were recovered from the fecal samples of hospitalized infants in (NICU), whereas all fecal samples obtained from non-hospitalized pa-

tients were negative. The majority of these isolates (62.5%) was significantly found (P=0.006) in feces of hospitalized femal infant as shown in **Table 2**.

Recent studies reported that nosocomial outbreaks caused by MDR *P. aeruginosa* are occurring around the world, and these outbreaks are frequently caused by clones of

P. aeruginosa producing metallo-betalctamases (MBLs), mostly VIM and IMP types. These resistance markers can be acquired either by chromosomal mutations or horizontal gene transfer [41-42]. Neonatal infection with *P. aeruginosa* is mostly acquired due to their underdeveloped immune system, especially when these infants are catheterized with intravascular catheters/devices and/or receiving parenteral nutrition, [43-44]. Almost all *P. aeruginosa* isolates in this study were resistant for one or more commonly used antibiotics in treatment of *Pseudomonas* infections, especially to carbapenems (meropenem and impenem) **(Table 3)**. This can be explained by the overexpression of efflux pumps that expel carbapenems and lead to carbapenem resistance [45]. However, all isolates were susceptible to colistin.

The present study demonstrates that both OXA-50 and TEM were the most frequent phenotypes found in *P. aeruginosa isolates*, followed by CTX-M, BlaVIM-2, whereas both blaIMP-15 and blaNDM-1 were not detected in all isolates. These findings are in agreement with a recent published study on *P. aeruginosa* isolates from respiratory tract of Jordanian patients [5].

The present study has detected that high percentage of our *P. aeruginosa* isolates (68.7%) were producers of KPC. This result should be considered serious since *KPC* genes found often in association with transferable plasmids and transposons, and can result in a rapid spread of these genes in Gram-negative opportunistic pathogens [46-47]. However, it is important to note that NDM-1 type carbapenemase is not detected in any of the

P. aeruginosa isolates

The majority of our fecal *P. aeruginosa* isolates were positive for some of the most important virulence factors as presented by ElastaseB and AlgD, and slightly in less rate for both extracellular protein toxins, ExoenzymeS and ExotoxinA. A previous Jordanian study has also shown that *P. aeruginosa* isolates from respiratory tract sources were mostly producers of same virulence factors [5].

The majority of our *P. aeruginosa* isolates produced pigments, either pyoverdin or pyocyanin. The redox-active pigment pyocyanin is responsible for the blue-green color characteristics of *P. aeruginosa,* and it is also required to express cell fatal cytotoxicity, while pyoverdine is a siderophore involved in iron acquisition, participate in biofilm formation, and its chelating activity may contribute in developing antibiotics resistance [13, 19].

Conclusion

This study provides important epidemiological data on antimicrobial resistance profiles, distribution of ESBLs and MBLs and presence of virulence factors among fecal *P. aeruginosa* isolates from infants during their hospitalized.

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